The functional diversity of red blood cells needs to be taken into account in future studies, which will increasingly require single-cell analysis approaches. The identified lysophosphatidic acid signalling cascade provides as a multicomponent system the potential for delays and gains and such provokes a tremendous variability. Heterogeneity in red blood cell responses is important for the basic understanding of red blood cell signalling and their contribution to numerous diseases, especially with respect to calcium influx and the associated pro-thrombotic activity.

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The Molecular Basis of Substrate Recognition by the E3 Ubiquitin Ligase Pellino

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The Pellino proteins are one of the several families of E3 ubiquitin ligases that direct ubiquitination events immediately following Toll and interleukin-1 receptor (TIR) activation. Polyubiquitination of known Pellino substrates, such as the interleukin-1 associated kinase (IRAK1), is a necessary step in mediating downstream signaling events that are responsible for eliciting a proper immune response. To elucidate Pellino's role in TIR signaling, we are investigating the molecular basis of Pellino substrate specificity. We previously determined the X-ray crystal structure of the human Pellino2 substrate recognition domain and found that it contains a non-canonical example of a well-characterized phosphothreonine (pT)-binding domain, the forkhead-associated (FHA) domain. In an attempt to determine the specific substrate-binding motif of Pellino2, we identified an IRAK1 truncation variant (aa 1-197, IRAK1-197) that interacts with Pellino2 in a phosphorylation dependent manner. Substitution of each threonine in IRAK1-197 with alanine identified T141 as the critical phosphorylated threonine on IRAK1-197 that Pellino2 specifically recognizes. A synthetic phosphopeptide corresponding to the sequence centered on T141 of IRAK1 (pT141 peptide) binds to Pellino2 with a Kd value in the 1 uM range; this data was assessed in a fluorescence polarization binding assay, and independently verified using isothermal titration calorimetry. Binding analyses of other mammalian Pellino isoforms (Pellino 1, 3A, and 3B) to the pT141 peptide reveals differences in binding affinities and specificities. These differences are hard to reconcile due to the high degree of sequence identity among the Pellino isoforms, and cannot be readily explained by the Pellino2 FHA domain crystal structure. Thus, to further explore the molecular basis of these differences, we are working towards determining the X-ray crystal structures of the other Pellino isoforms.

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Structural and Thermodynamic Insights into Bacterial Outer Membrane Lipid Signaling by the Innate Immune System

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Lipopolysaccharide (LPS) from bacterial outer membranes is a potent early indicator of microbial infection and the primary inducer of fatal septic shock syndrome. Its recognition by the immune system is carried out by Toll-like receptor 4 (TLR4) when associated with its co-receptor MD-2, an immunoglobulin-like protein. MD-2 adopts a characteristic ''beta-cup'' fold with a large hydrophobic cavity, and is able to bind a variety of lipid species. Subtle alterations in the structure of LPS derivatives can profoundly alter the resultant immunological response, hampering the rational design of TLR4 immunomodulators. To unravel the associated structure-activity relationships, we have performed long-timescale, all-atom molecular dynamics simulations and free-energy calculations of the isolated MD-2 co-receptor and the entire signaling-active receptor complex in the presence of a variety of LPS species, as well as an LPS membrane. Unbiased simulations revealed that the MD-2 cavity is highly conformationally flexible, identifying spontaneous switching between active signalling-competent and inactivated states dependent upon the presence of different ligands, leading us to propose a conserved receptor activation mechanism. To gain insights into the thermodynamic determinants of endotoxin recognition, extensive umbrella sampling has been applied to estimate the potential of mean force (PMF) for the binding of LPS molecules to MD-2 co-receptor. Strikingly, stronger binding to signalling-inactivate MD-2 were observed for antagonists, and conversely, stronger binding to active MD-2 for agonists. Comparison of this data to the first ever PMF calculated for extraction of LPS from a model of the bacterial outer membrane has revealed how MD-2 creates a ''membrane-like'' environment within its protein cavity, providing a mechanism for sensitive LPS recognition by the innate immune system.

3145-Pos Board B300

Molecular Mechanisms of Glutamate Receptor Activation and Regulation

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Neuronal signaling is based on the release and detection of glutamate within the synaptic cleft. Regulation of this signaling has implications in both learning and memory, while dysfunction is implicated in a variety of neurological disorders, including schizophrenia and depression. Glutamate in the synaptic cleft is detected by ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels. NMDA receptors (NMDARs) are a class of iGluRs that require the binding of both glycine and glutamate for receptor activation. We are concerned with the molecular mechanisms that govern the activity of NMDARs, both in terms of activation and regulation. Here, the free energy landscapes governing large-scale conformational transitions in the isolated ligand-binding domains (LDBs) of the NMDAR subunits GluN1, GluN2A, and GluN3A are computed for both apo and holo forms using umbrella sampling simulations. Agonist insertion shifts the bias to closed conformations to various degrees for the different LBDs. We compare the structural elements that determine the stability of the open and closed states of NMDARs with those in a bacterial homologue GluR0. In addition we present structural data examining the interaction between a subclass of AMPA receptors (AMPARs), another class of iGluRs, and isolated elements of protein 4.1, a chaperone that regulates AMPAR insertion into the plasma membrane.

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Non-Genomic Progesterone Signalling in Human Sperm

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In human sperm, CatSper (cation channel of sperm) Ca^{2+} channels control the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and, thereby, the swimming behaviour. By patch-clamp recordings from human sperm and kinetic Ca^{2+} fluorimetry, we have shown that CatSper is directly activated by the female sex hormone progesterone; cells surrounding the oocyte release progesterone into the oviduct to assist sperm for fertilization. The rapid Ca^{2+} influx evoked by progesterone has been implicated in sperm chemotaxis, hyperactivation, and acrosomal exocytosis. We studied progesterone-evoked voltage responses in human sperm with the kinetic stopped-flow technique, using electrochromic voltage-sensitive dyes. We show that progesterone evokes instantaneous changes in membrane voltage (Vm) governed by activation of CatSper and another ion channel present in human sperm. We elucidated the molecular identity of this channel by patch-clamp recordings from human sperm.

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Protein-Protein-Protein Interactions in Membranes Measured by Triple Cross-Correlation of Confocal Images

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Protein-protein interactions in binary complexes have been measured successfully and quantitatively by fluorescence correlation spectroscopy in solution (Elson, BJ 101, 2855-2870 (2011)) and image correlation spectroscopy on cell surfaces (Kolin and Wiseman, Cell Biochem Biophys 49, 141-164 (2007)). These tools fail, however, to provide information about ternary complexes i.e. about **protein-protein-protein** interactions. It has been known for some time that higher order moments or correlations contain the relevant information (Palmer and Thompson, BJ 52, 257-270 (1987); Heinze, Jahnz, and Schwille, BJ 86, 506-516 (2004)) but it is only recently that triple correlation functions of ternary complexes have been measured in solution (Ridgeway, Millar, and Williamson, J. Phys Chem B 116, 1908-1919 (2012) and PNAS 109, 13614-13619 (2012)). Our present work shows how complete and quantitative information can be obtained for ternary complexes of membrane proteins from three confocal images from each of three distinctly labeled protein species by employing a combination of image correlation spectroscopy, image cross-correlation spectroscopy, image triple auto-correlation spectroscopy and image triple cross-correlation spectroscopy.

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Detailed Analysis of Phospholipase C-β Activity in Living Cells

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Phospholipase C-β (PLC-β) catalyzes the hydrolysis of the plasma membrane (PM) lipid phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$), thereby reducing the PM concentration of its precursor PI(4)P. A recent report proposed independent $PI(4,5)P_2$ and PI(4)P PM pools with essential physiological functions, but it remains unknown whether PLC-β targets to these pools differentially.

Here, we investigated the time course and specificity of PLC-β-dependent phospholipid depletion in living CHO cells using genetically-encoded phospholipid sensors, total internal reflection fluorescence microscopy and whole cell patch clamp. PLC-β mediated $PI(4,5)P_2$ hydrolysis was detected reliably using KCNQ (Kv7)-mediated K^+ currents, the $PI(4,5)P_2$ reporters $PLC\delta_1$-PH and Epsin ENTH, but not with the C-terminus of Tubby protein. Analogous experiments showed that activation of PLC-β did not reduce the concentration of PI(4) detected with the PI(4)P-specific reporter Osbp-PH. When PLC-β3 was heterologously overexpressed, Tubby-Cterm and Osbp-PH reported on the PLC-β-induced changes of $PI(4,5)P_2$ and PI(4)P, respectively.

In summary, we present detailed real-time analysis of PLC-β activity in living cells. Our findings indicate that phospholipid sensors may detect different phospholipid pools that are accessible to PLC-β differentially. Hence, this work supports the presence of functional phospholipid pools in living cells.

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3149-Pos Board B304

Regulation of CAMP Compartmentation by Membrane Microdomains

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The role of different membrane domains in the compartmentation of cAMP signaling was investigated using the FRET-based biosensor Epac2-camps. The MyrPalm sequence from Lyn kinase and the CAAX sequence from Rho GTPase were used to target this probe to lipid raft (Epac2-MyrPalm) or non-lipid raft (Epac2-CAAX) domains, respectively. Confocal imaging established that both probes were targeted to the plasma membrane in HEK293 cells. FRAP analysis demonstrated that depletion of membrane cholesterol altered both the recovery half-time and the mobile fraction of Epac2-MyrPalm but not Epac2-CAAX, confirming that each probe was targeted to the correct microdomain. FRET responses of these probes were then used to monitor relative changes in cAMP activity associated with lipid raft and non-raft domains. The results demonstrated that basal cAMP activity is significantly higher in non-raft domains. This was supported by the fact that the maximal increase in cAMP over baseline following agonist stimulation was significantly smaller for Epac2-CAAX than it was for Epac2-MyrPalm, consistent with the idea that the probe was partially activated by a higher basal level of cAMP associated with non-lipid raft domains. In addition, inhibition of adenylyl cyclase activity with MDL 12330A reduced basal cAMP activity detected by Epac2-CAAX but not Epac2-MyrPalm. Responses detected by Epac2-CAAX were also more sensitive to direct activation of adenylyl cyclase by forskolin, but less sensitive to inhibition of type 4 phosphodiesterase activity by rolipram. These results indicate that there are diffusionally-restricted pools of cAMP associated with different membrane microdomains under basal conditions. The higher basal cAMP activity associated with non-lipid raft domains can be explained by differences in basal adenylyl cyclase and phosphodiesterase activity.

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Integrating High-Resolution Bioimaging Techniques to Unravel How Membrane Lipids Influence Nanoscale Organization and Lateral Mobility of Adhesion Receptors

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A central, but still unresolved question regarding the function of integrins is how these adhesion receptors regulate both their conformation and dynamic nanoscale organization on the membrane to generate adhesion-competent microclusters upon ligand binding. By superresolution nanoscopy, we recently showed that in quiescent monocytes, LFA-1 preorganizes in ligand-independent nanoclusters proximal to nanoscale raft components (1,2). Furthermore, to dissect the relationship between conformational state, lateral mobility, and microclustering we exploited the high spatial (nanometer) accuracy and temporal resolution of single dye tracking and found that LFA-1 nanoclusters are primarily mobile on the cell surface with a small (ca. 5%) subset of conformational-active LFA-1 nanoclusters preanchored to the cytoskeleton (3). Lateral mobility resulted crucial for the formation of microclusters upon ligand binding and for stable adhesion under shear flow. Ongoing investigation in our laboratory points towards the importance of a specific lipid composition of the membrane nano-environment in modulating LFA-1 biophysical properties which eventually regulate the onset of leukocyte adhesion. Since several (patho)physiological stimuli can alter either temporarily or permanently the plasma membrane lipid composition, our studies offer a novel framework to understand integrin regulation via the lipid nanoenvironment.

(1) Van Zanten et al PNAS 2009.
(2) Van Zanten et al PNAS 2010.
(3) Bakker et al PNAS 2012.

3151-Pos Board B306

Visualizing Ghrelin Receptor through Genetically Encoded Labeling for Monitoring the Single-Molecule Conformational Dynamics

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The ghrelin receptor (GhR) is a class A G protein-coupled receptor (GPCR) involved in the entero-endocrine signaling systems that regulates food intake and energy homeostasis. The GhR is noted for its unusually high basal constitutively activity. GhR is a potential drug target for ''diabesity'' syndromes, and the interaction between GhR and its endogenous peptide ligand, ghrelin, has been intensively studied. However, there is only a limited understanding of GhR pharmacology and its molecular mechanism of signal transduction. Using well-established amber codon suppression technology and state-of-the-art single-molecule techniques, we are developing tools to monitor directly differential conformational dynamics of GhR in the presence and absence of its binding partners, including ligands, G proteins, or other GPCRs. For example, we are preparing single-site and double-site fluorescently labeled GhR and a series of labeled ghrelin analogues. These engineered receptors can be studied in cell-based systems or reconstituted in NABBs (Nanoscale Apolipoprotein Bound Bilayers) after purification. These types of approaches will enable us to better understand the complexity of GhR signaling in the neuro-endocrine system, providing insights to design specific drugs for targeting fine-tuned signal pathways involved in metabolic disorders like obesity and diabetes.

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TGF-Beta and Bmp Receptors: Distinct Modes of Oligomeric Interactions and Implications for Signaling

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Transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) signal *via* two Ser-Thr kinase receptors, type I type II (TβRI and II, BMPRI and II for the TGF-β and BMP receptors, respectively), which appear at the cell surface as monomers, homomeric and heteromeric complexes. their extracellular domains (EDs) complexed with ligands were shown to form heterotetramers. However, the interaction dynamics among the full-length receptors in live cell membranes, the domains involved, and the potential roles of receptor homodimerization were largely unexplored. Using patch/FRAP and computerized immunofluorescence co-patching of epitope-tagged receptors [wild-type (wt) or mutants] in live cells, we show that the oligomerizaiton dynamics are distinctly different for the two receptor systems. For TGF-β receptors, we find clear differences between TβRII and TβRI oligomeric interactions: (1) the homodimerization of TβRII, but not TβRI, depends on a cytoplasmic juxtamembrane region; and (2) TβRI/TβRII hetero-oligomerization depends on the cytoplasmic domain of TβRI and on a C-terminal region of TβRII, distinct from the region involved in TβRII homodimerization. TGF-β1 binding mildly elevated TβRII homodimerization, and strongly enhanced TβRI/TβRII heteromeric complex formation. Notably, both homomeric and heteromeric TGF-β receptor complexes were stable on the patch/FRAP timescale (minutes).

In contrast, the BMP receptors display stable interactions on the same timescale only for homomeric complexes, while the heterocomplexes are transient. Interestingly, the BMPR heterocmplexes appear to form at the expense of homodimers, and stabilization of BMPRII/BMPRIb heteromeric (but not homomeric) complexes by IgG binding elevates phospho-Smad formation both without and with BMP-2. Based on these findings, we propose two mechanisms that can suppress the tendency of preformed BMP receptor hetero-oligomers to signal without ligand.